

Neuregulin-1 Promotes Myocardial Angiogenesis in Acute Myocardial Infarction Through Regulating PI3K-AKT-eNOS and VEGF/VEGFR2 Signal Pathways

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Research Article

Keywords: Neuregulin-1, acute myocardial infarction, angiogenesis

Posted Date: January 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-138431/v1>

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Abstract

Background: Myocardial angiogenesis is central to the recovery of acute myocardial infarction (AMI). Neuregulin-1 (NRG-1) plays a critical role in cardiac function, although its role in myocardial angiogenesis is still unclear. The aim of this study was to investigate the effects of NRG-1 in myocardial angiogenesis in a rat model of AMI, and elucidate the underlying mechanisms.

Methods: AMI was induced by a single ligation of left anterior descending coronary artery, followed by intravenous injection of recombinant human NRG-1 or normal saline for 8 consecutive days. The cardiac function indices were measured using the catheter MPA cardiac function analysis system. Histo-pathological changes were observed by HE. Microvessel density (MVD) was measured by CD31 and α -SMA immunostaining. The expression levels of other proteins were assessed by Western blotting.

Results: NRG-1 improved cardiac function and alleviated myocardial damage induced by AMI. Compared to the sham-operated group, the capillary density and arteriole density increased after AMI ($P < 0.05$), and were augmented by NRG-1 which also significantly increased the left ventricular function ($P < 0.05$). Furthermore, Compared with sham group, PI3K-AKT-eNOS signaling was decreased significantly ($P < 0.05$) whereas VEGF/ VEGFR2 signaling was significantly increased ($P < 0.05$) in AMI group and both of them were further upregulated by NRG-1 ($P > 0.05$).

Conclusion: NRG-1 improved cardiac function and promoted myocardial angiogenesis post AMI by up-regulating VEGF and activating the PI3K-Akt-eNOS pathway.

Introduction

Acute myocardial infarction (AMI) is the leading cause of mortality and disability associated with cardiovascular diseases worldwide [1]. The current method for treating AMI is revascularization through percutaneous coronary intervention (PCI) or coronary artery bypass grafting, which however are limited by coronary artery diffuse stenosis [2] and iodinated contrast media hypersensitivity [3], resulting in the high long-term mortality of patients post-AMI [4]. Angiogenesis, or the generation of new blood vessels from pre-existing capillaries, is a vital compensatory mechanism in myocardial ischemia which supplies the ischemic tissue with blood [5]. Therefore, therapeutic angiogenesis is a viable strategy to improve reperfusion and cardiac function after myocardial infarction [6], and angiogenesis induction in AMI has gained considerable attention in recent years [7].

Neuregulin-1 (NRG-1) is a paracrine growth factor synthesized by the endocardium and microvascular endothelium, and is involved in adult heart maintenance [8]. Studies show that NRG-1 increases survival of cardiomyocytes [9], reduces oxidative stress [10] and induces cardiomyocyte proliferation post heart injury [11]. A recent phase II clinical trial revealed that the short-term administration of recombinant human NRG-1 can improve cardiac function after heart failure [12]. In addition, there are reports indicating a pro-angiogenic role of NRG-1 as well. For instance, Ebner et al. found that remote application of recombinant NRG-1b protected the heart against early reperfusion injury without affecting

hemodynamics [13]. Similarly, Hedhli et al. reported that NRG-1 released by endothelial cells is necessary for arteriogenesis and angiogenesis, and the administration of exogenous NRG-1 can promote both processes [14]. However, the precise role of NRG-1 in myocardial angiogenesis remains to be elucidated.

In a previous study, we found that NRG-1 promoted myocardial angiogenesis in a rat model of diabetic cardiomyopathy along with other angiogenic factors [15]. Phosphatidylinositol 3 kinase (PI3K) signal pathway is closely related to angiogenesis and vascular endothelial growth factor (VEGF) is the key and strongest angiogenic factor to induce angiogenesis. Law et al. reported a mechanistic link between NRG-1 and the PI3K-AKT signaling pathway in patients with schizophrenia [16]. In addition, Ferrara et al found that NRG-1-induced angiogenesis can be maintained by both VEGF-dependent or independent pathways [17]. We hypothesized that the mechanisms of NRG-1 promoting angiogenesis in AMI may be associated with VEGF or PI3K-AKT-eNOS signaling pathway. In this study, we explored the effects of NRG-1 in myocardial angiogenesis and elucidated the underlying mechanisms in a rat model of AMI.

Materials And Methods

Induction of AMI and treatment regimen

A total of 33 male Sprague–Dawley rats (8–10 weeks old and weighing 250–280 g) were obtained from the Experimental Animal Center of Guangxi Medical University. All animal experiments were approved by the Animal Care and Use Committee of the Guangxi Medical Institute (Protocol ID#: Gui 2014-002), and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. The animals were randomly divided into the sham-operated, and the untreated and NRG-1-treated AMI groups. The AMI model was established as previously described [18]. Briefly, the rats (n = 24) were anesthetized with an intraperitoneal injection of 4% chloral hydrate solution (1 ml/100 g body weight), and then rapidly intubated and mechanically ventilated (tidal volume, 4 ml/100 g body weight; ventilation rate 80 strokes/min). The chest was then opened gently by left thoracotomy at the 3rd intercostal space, and the left anterior descending coronary artery was ligated 1–2 mm from the tip of the left atrial appendage using a 6–0 polyester suture. ST-segment elevation and pathological Q waves were confirmed by electrocardiography. The same procedure was followed for the sham-operated rats (n = 9) except for the ligation step. Recombinant human NRG-1 (rhNRG-1) was administered daily for 8 days at the dose of 10 g/kg via the intravenous route, while the sham-operated and untreated AMI rats were injected with the same volume of normal saline.

Evaluation of cardiac function

The hemodynamic and cardiac functional parameters were evaluated using a catheter MPA cardiac function analysis system as described previously [19]. Eight days after the operation, all rats were weighted and anesthetized with 4% chloralhydrate (1 ml/100 g). After intubation and mechanical ventilation at stable tidal volume (4 ml/100 g), left anterolateral longitudinal thoracotomy was performed to expose the apex, and a catheter was inserted into the left ventricle and connected to the analysis system. Stable conditions were established, and the left ventricular systolic pressure (LVSP), left

ventricular end-diastolic pressure (LVEDP) and maximum increase and decrease rates of left ventricular pressure ($\pm dp/dt_{\max}$) were detected and recorded.

Cardiac tissue harvest

After evaluating the cardiac function, heart tissues were harvested and weighted. The tissues from the paler infarct regions and peri-infarct regions within 5 mm of the former were collected from the left ventricle, and divided into two parts. One portion of each was snap frozen in liquid nitrogen and stored at -80°C , and the other was fixed in 10% neutral buffered formalin, embedded into paraffin and cut into 5 μm thick section.

Hematoxylin-eosin (HE) staining

Cardiac tissue sections were dewaxed in xylene for 20 min, and dehydrated through an ethanol gradient. The sections were then immersed sequentially in hematoxylin (5 min), 1% hydrochloric acid (30 s) and 1% eosin -alcohol (5 min), followed by another round of gradient alcohol dehydration. Five random fields from each section were evaluated under 200X magnification by two investigators who were blinded to the grouping. The area of the diseased myocardium relative to that of the entire myocardium was calculated in terms of inflammation, ischemia and necrosis, and the samples were histo-pathologically scored as follows: 0 – absence of the above signs, 1 – < 25% of the myocardium, 2 – 25%-50% of the myocardium, 3 – 50%-75% of the myocardium, and 4 – > 75% of the myocardium showing the above signs.

Immunohistochemistry

Myocardial cross sections were cleared and dehydrated as described, and treated with 3% hydrogen peroxide to quench the endogenous peroxidases. After incubating with rabbit anti- α -SMA (Abcam, 1:200 dilution) and rabbit anti-CD31 (Abcam, 1:100 dilution) antibodies for 2–3 hours, the sections were then treated with real time MaxVision, followed by the secondary antibody (DAKO, 1:14000) for 90 minutes. The sections were developed using DAB solution and counterstained with hematoxylin. Microvessels in the infarct and peri-infarct regions were evaluated in two separate slides from at least 6 random fields under 400X magnification. Microvessel density (MVD) was calculated by dividing the number of microvessels by the field area.

Western blotting

Myocardial tissues were homogenized with RIPA buffer supplemented with protease inhibitor cocktail, and the protein concentration of the lysates was measured using ND-2000 UV spectrophotometer or BCA Protein Assay Kit. Equal amounts of protein per sample were resolved by SDS-PAGE, and the bands were transferred to nitrocellulose membranes. After blocking the membranes with 5% bovine serum albumin in TBS-Tween buffer for 1 hour at room temperature, they were incubated overnight with antibodies against VEGF (Novusbio, 1:1000), VEGFR2 (Abcam, 1:800), AKT (Cell signaling; 1:1000), eNOS (Cell signaling; 1:1000), p-AKT (Cell signaling; 1:2000), p-eNOS (Genetex, 1:600), and PI3K (Cell signaling, 1:1000) at 4°C . The blots were washed thrice and then incubated with horseradish peroxidase-conjugated secondary

antibody (DAKO, 1:14000) for 2 hours at 4°C. Enhanced chemiluminescence detection system was used to develop the positive bands, and their densities were analyzed using Image-Pro Plus6.0 software.

Statistical analysis

The continuous variables were expressed as the mean \pm SD. All data were analyzed using the SPSS 17.0 statistic software package by two researchers blinded to the study. Two-sample t-test was used to compare two groups, one-way analysis of variance for multiple groups, and least significant difference t-test for pair-wise comparison between groups. P values less than 0.05 were considered statistically significant.

Results

AMI model was successfully established

The myocardial tissues below the artery ligation line appeared pale during surgery, and 8 days later, the infarcted region was distinctly pale and thin compared to the healthy tissues (Fig. 1A). While all sham-operated animals survived during the experiment, 7 of the 33 rats that underwent arterial ligation were dead within 24 h of surgery. Furthermore, the levels of phosphorylated ErbB receptors increased in the myocardium of healthy animals following administration of rhNRG-1, indicating high efficacy of the latter (Fig. 1B). The indices of cardiac function, including LVSP, +dp/dtmax and -dp/dtmax, were significantly reduced while LVEDP was increased in the AMI group compared to the sham-operated group, and restored by rhNRG-1 (Fig. 1C and Table 1).

NRG-1 alleviated myocardial injury and restored angiogenesis post AMI

As shown in Fig. 1D, the myocardial tissues of the sham-operated animals were arranged in an orderly manner, whereas massive infiltration of inflammatory cells was seen in the cardiac tissues following AMI. NRG-1 treatment significantly repaired the myocardial injury, as compared to that of the untreated AMI rats .

We next analyzed the *in situ* expression of CD31 and α -SMA as the respective markers of capillary and arteriole densities [20, 21]. Compared to the myocardium of the sham-operated animals, that of the AMI group showed similar capillary density in the infarct region but significantly higher density in the peri-infarct region. NRG-1 treatment on the other hand significantly increased the capillary density in the infarct region as well (Fig. 2). Furthermore, the arteriole density was significantly increased in both the infarct and peri-infarct regions of the AMI group, and was augmented further by NRG-1 (Fig. 3). Taken together, NRG-1 treatment not only improved the myocardial architecture but also arteriole density post AMI .

NRG-1 promotes angiogenesis via the VEGF/VEGFR2 and PI3K/AKT/eNOS signaling pathways

To elucidate the mechanisms underlying the pro-angiogenic effects of NRG-1, we next examined the expression levels of the VEGF/VEGFR2 and PI3K-AKT-eNOS signaling pathways. Compared to the sham-operated group, VEGF was slightly upregulated in the infarct and peri-infarct regions of the AMI group, whereas VEGFR2 expression levels were slightly increased in the infarct region and unaltered in the peri-infarct region. In contrast, both VEGF and VEGFR2 were significantly upregulated in the infarct region and only slightly increased in the peri-infarct region of the NRG-1-treated animals (Fig. 4). Furthermore, AMI resulted in a significant decrease in the levels of PI3K and p-AKT in the infarct region, and a minor reduction in the peri-infarct region. NRG-1 administration significantly upregulated PI3K, p-AKT and p-eNOS in the infarct as well as in the peri-infarct region (Fig. 5). Taken together, the pro-angiogenic effects of NRG-1 are likely mediated via activation of the VEGF/VEGFR2 and PI3K/AKT/eNOS pathways (Fig. 6).

Discussion

We report for the first time that NRG-1 improves the density of capillaries and arterioles in the different ischemic regions post AMI. In addition, NRG-1 also improved left ventricular function after infarction and inhibited myocardial fibrosis in a rat model of AMI. Mechanistically, the protective effects of NRG-1 were mediated via activation of the proangiogenic VEGF/VEGFR2 and PI3K/AKT/eNOS pathways in the cardiac tissues.

The rat model of AMI has been established previously [18]. AMI results in myocardial cell apoptosis, cardiac inflammation and myocardial fibrosis that adversely affect cardiac systolic and diastolic functions [22]. It eventually leads to ventricular wall thinning, left ventricular cavity expansion and cardiac ventricular remodeling, all of which increase the risk of heart failure [23]. We found that NRG-1 significantly inhibited myocardial collagen deposition post-AMI and improved cardiac function. The NRG-1/ErbB system is crucial for adapting to cardiac demands, and its disruption reduces tolerance to myocardial ischemia [24]. NRG-1 administration also improves cardiac function in animal models of ischemic heart disease, dilated cardiomyopathy and viral cardiomyopathy [8]. At the cellular and molecular level, it improves cardiomyocyte survival [25], ameliorates myofilament injury [26], prevents mitochondrial dysfunction [27], enhances reparative inflammatory response [28], and increases calcium intake in the sarcoplasmic reticulum [29].

Our study showed a pro-angiogenic effect of NRG-1 in an animal model of AMI, which was the likely basis of improved cardiac function. This is consistent with Hedhli et al. who reported that exogenous injection of NRG-1 improved blood flow in an ischemia model [14], as well as our previous study wherein we found that NRG-1 increased the levels of angiogenic factors *in vitro* and promoted myocardial angiogenesis *in vivo* [15]. In the current study, NRG-1 treatment increased capillary density in peri-infarct region and arteriole density in the infarct region. Angiogenesis, or the formation of new blood vessels from pre-existing vascular beds, is activated during ischemia [30]. The ischemic and hypoxic conditions in the core infarct regions urgently require vascular compensation, resulting in increased collateral arterioles following NRG-1 treatment. However, since the injury in the peripheral region is relatively minor, NRG-1 promotes the formation of new capillary cavities that act as reserves of mature blood vessels.

Angiogenesis is a complex process that requires the participation of various angiogenic factors, pathways and cells [31, 32]. a rat model of diabetic cardiomyopathy along with other angiogenic factors [15]. PI3K-AKT-eNOS signaling cascade is closely related to angiogenesis and VEGF is the key and strongest angiogenic factor to induce angiogenesis. We found that NRG-1 significantly activated this pro-angiogenic pathway in both the infarct and peri-infarct regions, which is consistent with previous studies showing that NRG-1 induces angiogenic factors in various tissues and cells. For instance, NRG-1 β promotes glucose uptake in neonatal rat cardiomyocytes via the PI3K/Akt pathway [33], and induces VEGF secretion by endothelial cells [34]. The PI3K-Akt-eNOS pathway lies downstream of VEGF/VEGFR [35, 36], although the relationship is more complex *in vivo*. Nevertheless, our results clearly show that they are critical to myocardial angiogenesis post AMI.

Our study has several limitations that should be acknowledged. First, although the hemodynamic parameters improved significantly following NRG-1 treatment, the actual infarct size was not measured. Second, the use of α -SMA as a marker of angiogenesis is controversial despite recent studies showing that activated fibroblasts express higher levels of α -SMA following myocardial infarction [5, 36]. Third, the effect of NRG-1 on the angiogenic pathways was not validated by genetic or pharmacological inhibition of the said factors. Finally, it remains to be clarified whether NRG-1 regulates other angiogenic pathways in AMI.

In conclusion, NRG-1 improved cardiac function and promoted myocardial angiogenesis in a rat model of AMI by up-regulating VEGF and activating the PI3K-Akt-eNOS pathway.

Abbreviations

AMI

acute myocardial infarction;NRG-1:Neuregulin-1;LVEDP:left ventricular end-diastolic pressure; LVSP:left ventricular systolic pressure; $\pm dp/dt_{max}$:decrease rates of left ventricular pressure;MVD:Microvessel density ;PI3K:Phosphatidylinositol 3 kinase;VEGF:vascular endothelial growth factor

Declarations

Acknowledgements

Not applicable.

Authors' contributions

CG conceived and designed the experiments; XM and CQW performed the experiments and wrote the paper; ZYZ, LL analyzed the data. Xiao Ma and Chengqiang Wu made the same contribution to the study. All authors have read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding

This study was supported by the National Natural Science Foundation of China (grant no. 81460063).

Ethics approval and consent to participate

This investigation was approved by the Animal Care and Use Committee of the Guangxi Medical Institute (Protocol ID#: Gui 2014-002). the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Competing interests

The authors declare that there is no conflicts of interest.

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Tables

Table 1. The measurements of hemodynamic and functional parameters

Group	Sham(n = 9)	AMI(n = 9)	NRG-1(n = 8)
HR(beats/min)	459.83 ± 47.73	440.00 ± 36.36	487.40 ± 76.75 1.59 0.27
+dp/dt _{max} (mmHg/s)	6342.71 ± 541.42	4404.67 ± 853.51*	5231.53 ± 989.89*#
-dp/dt _{max} (mmHg/s)	4627.82 ± 265.97	3398.23 ± 195.63**	4168.52 ± 391.41##
LVSP(mmHg)	126.83 ± 13.71	80.20 ± 8.78**	104.91 ± 9.01*#
LVEDP(mmHg)	4.51 ± 2.11	10.69 ± 4.07**	4.41 ± 3.02*#

Note. Measurement data were expressed as mean ± standard deviation, and comparisons of which among multiple groups were assessed by one-way analysis of variance. NRG-1: Neuregulin-1; HR: heart rate; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end-diastolic pressure; +dp/dt_{max}: maximum increasing rates of left ventricular pressure; -dp/dt_{max}: maximum decreasing rates of left ventricular pressure.

* $P < 0.05$, ** $P < 0.01$ versus the normal groups;

$P < 0.05$, ## $P < 0.01$ versus the AMI groups

Figures

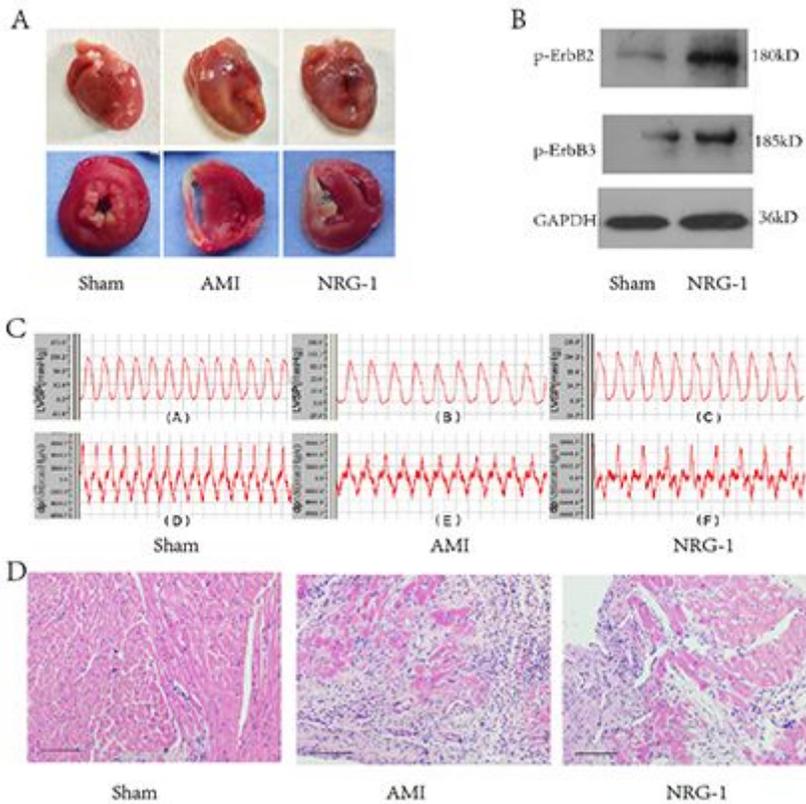


Figure 1

A. The cross-sectional view of myocardial tissues in the three groups. B. The expression levels p-ErbBR2 and ErbBR3 in normal rats before and after injection of NRG-1. C. Left ventricular LVSP, +dp/dt and -dp/dt values in the sham-operated (a and d), AMI (b and e) and NRG-1 (c and f) groups. D. Representative HE stained images of left ventricles in each group . AMI - acute myocardial infarction; NRG-1 - neuregulin-1; LESP - left ventricular systolic pressure; +dp/dtmax - maximum increasing rates of left ventricular pressure; -dp/dtmax - maximum decreasing rates of left ventricular pressure.

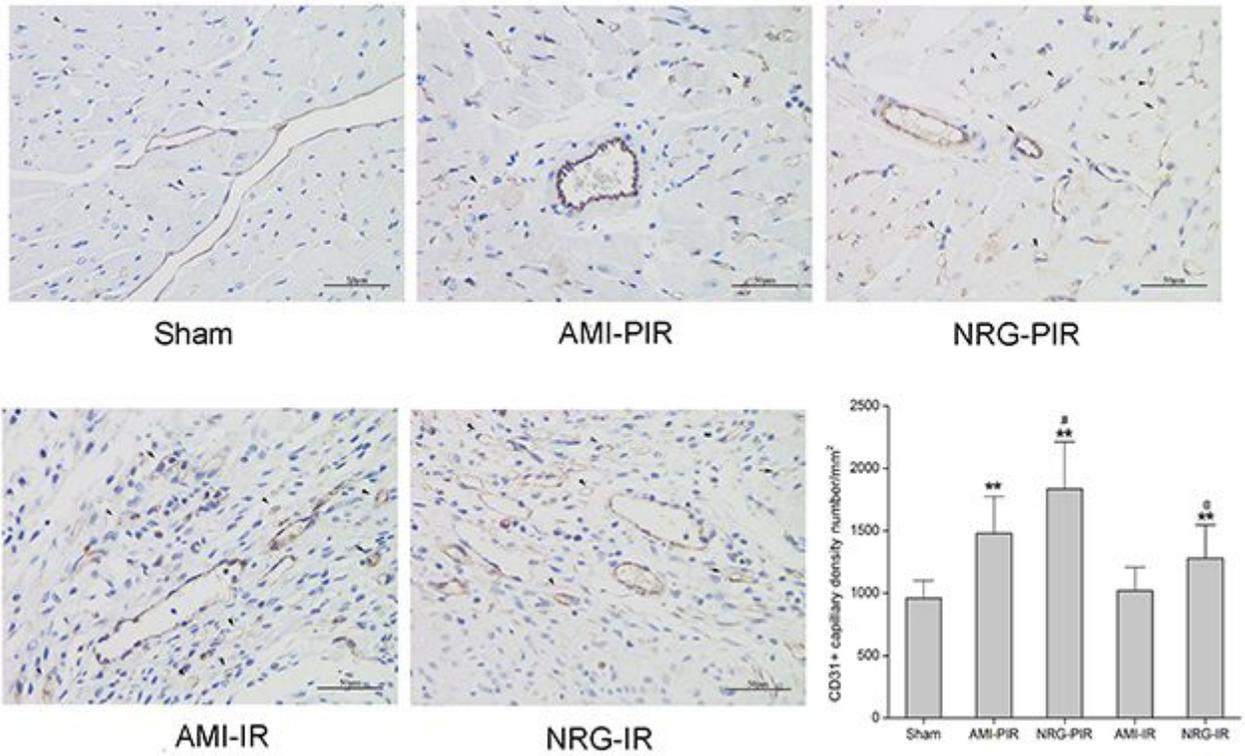


Figure 2

Representative IHC images of CD31 in myocardial tissues of three groups and respective capillary densities (n=8, bar=50µm). AMI - acute myocardial infarction; NRG-1 - neuregulin-1; PIR - peri-infarct region; IR - infarct region. Magnification – 400x. CD31+ capillary endothelial cells are stained brown and myocardial sections are counterstained with hematoxylin (nucleus is dark blue). *: P<0.01 vs control group; #: P< 0.05 vs AMI group.

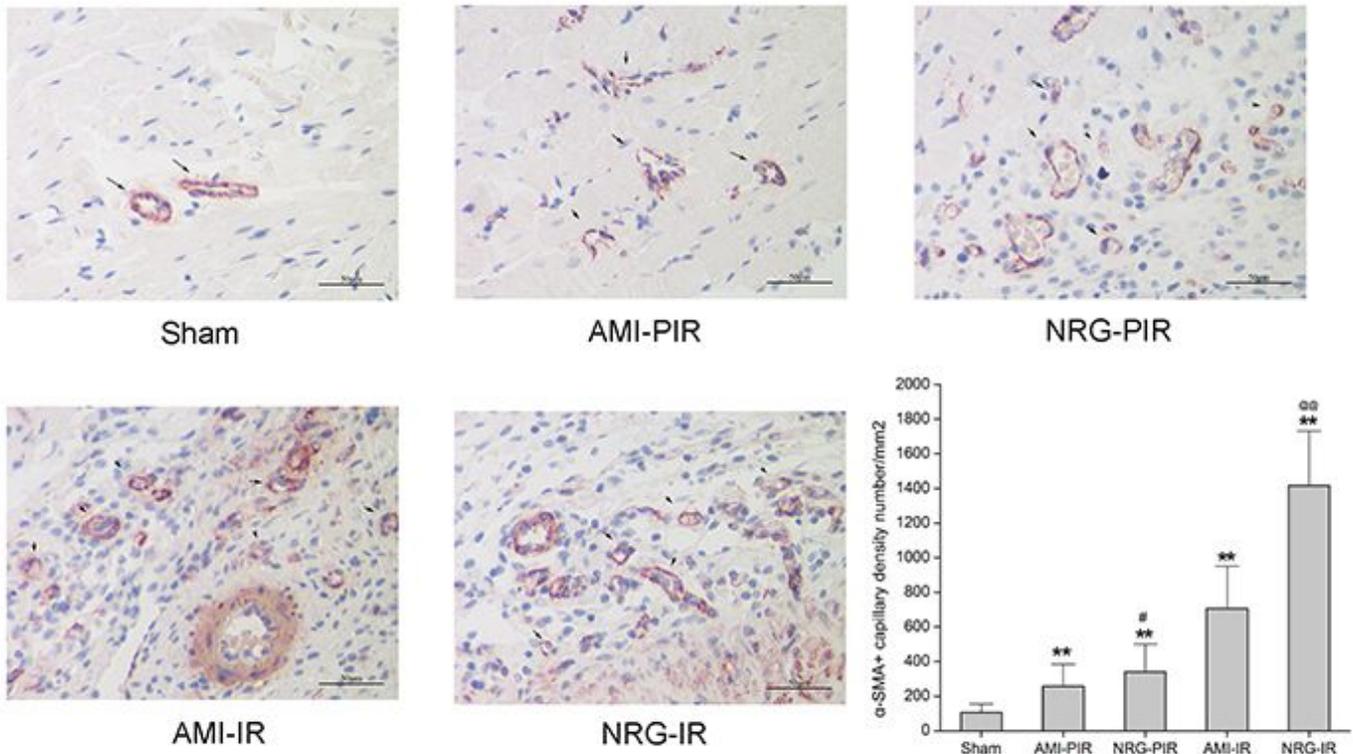


Figure 3

Representative IHC images of α -SMA in myocardial tissue of three groups and respective arteriole densities (n=8, bar=50 μ m). AMI - acute myocardial infarction; NRG-1 - neuregulin-1; PIR - peri-infarct region; IR - infarct region. Magnification – 400x. The α -SMA+ arteriole endothelial cells are stained light red and myocardial sections are counterstained with hematoxylin (nucleus is dark blue). *: P<0.01 vs control group; #: P< 0.05 vs AMI group.

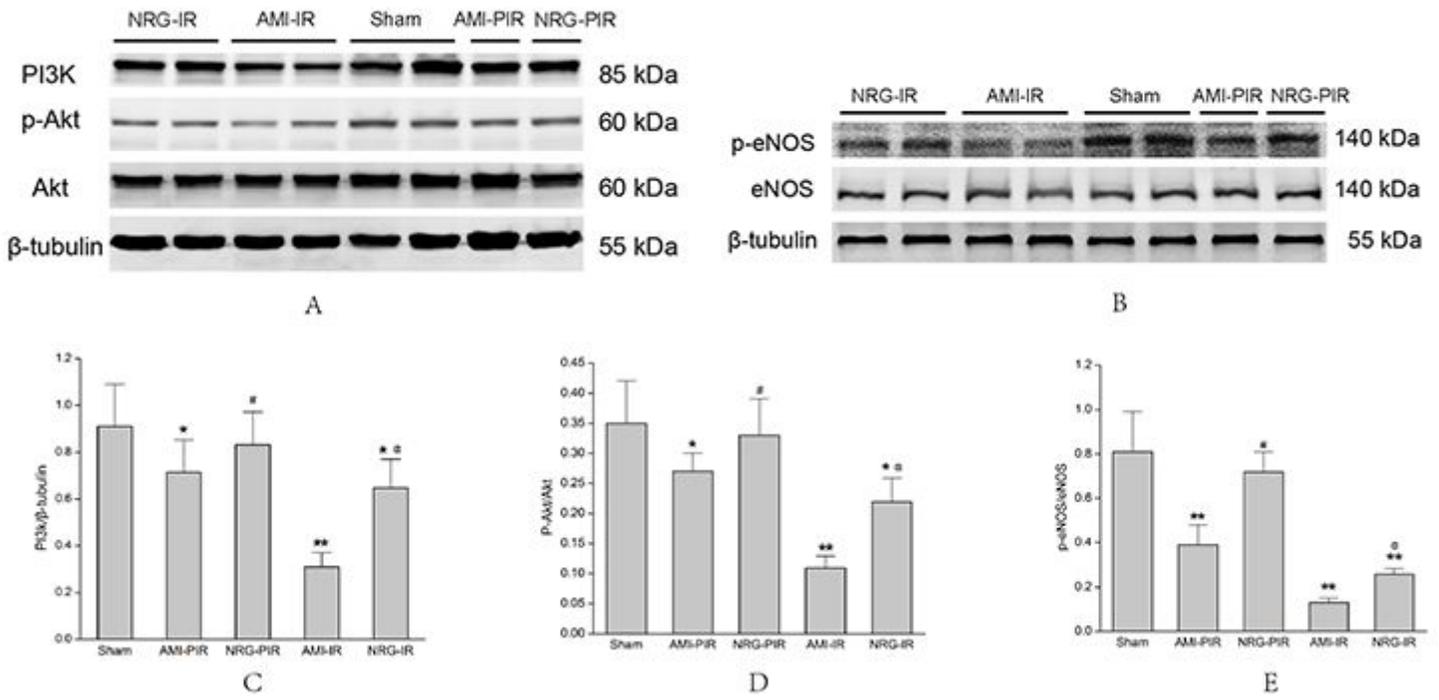


Figure 4

Representative immunoblots showing expression of PI3K-AKT-eNOS mediators in the three groups 8 days post-MI (n=8). Expression levels of (A) PI3K, AKT, p-AKT, (B) eNOS and p-eNOS. Comparison of (C) PI3K, (D) p-AKT/AKT and (E) p-eNOS/ e-NOS levels among three groups. AMI - acute myocardial infarction; NRG-1 - neuregulin-1; PIR - peri-infarct region; IR - infarct region.

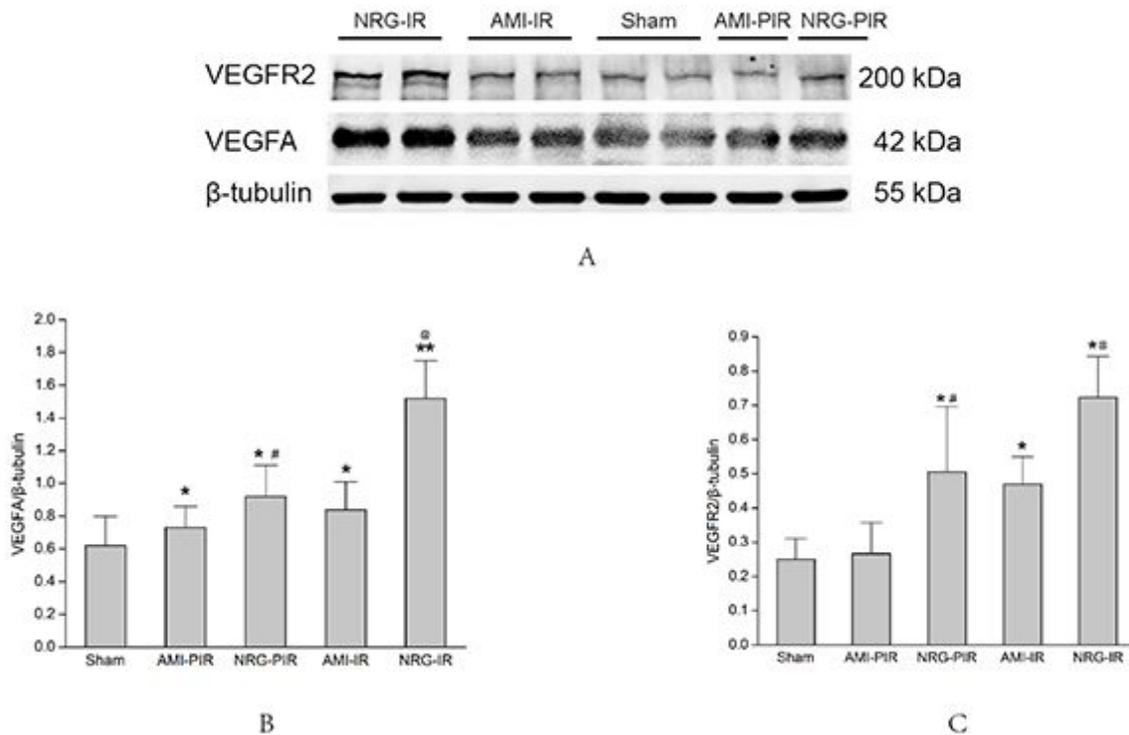


Figure 5

Representative immunoblots showing VEGF/VEGFR2 levels in the three groups 8 days post-MI (n=8). A. The expression of VEGF and VEGFR2. Comparison of (B) VEGF and (C) VEGFR2 levels among three groups. AMI - acute myocardial infarction; NRG-1 - neuregulin-1; PIR - peri-infarct region; IR - infarct region.

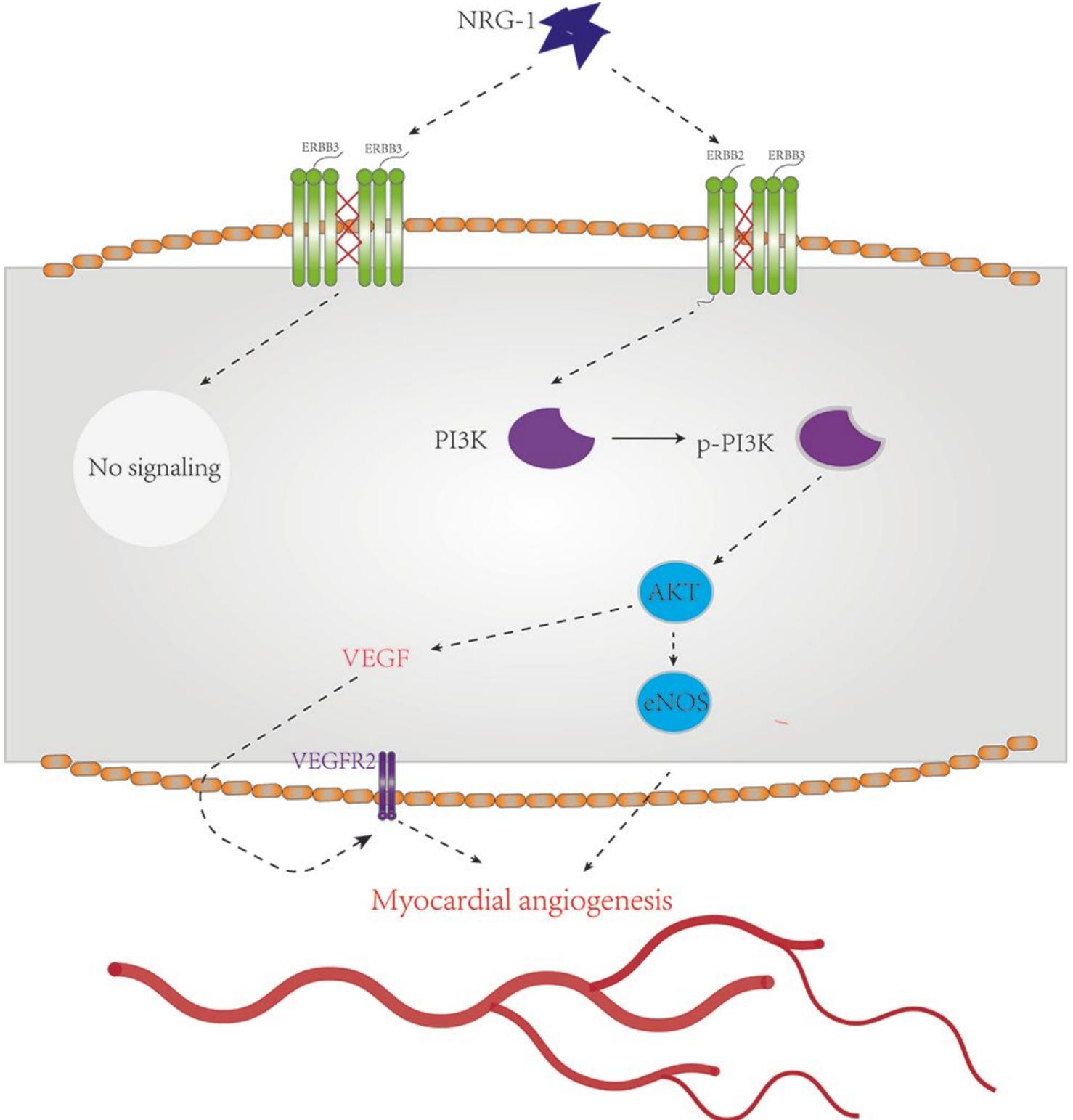


Figure 6

Taken together, the pro-angiogenic effects of NRG-1 are likely mediated via activation of the VEGF/VEGFR2 and PI3K/AKT/eNOS pathways (Fig. 6).